Effect of postmortem storage on μ-calpain and m-calpain in ovine skeletal muscle[1,2]

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ABSTRACT: Casein zymography was used to determine the effect of postmortem storage on the proteolytic activity of μ-calpain and m-calpain in lamb longissimus. Casein zymography assays were conducted on crude muscle extracts (only one centrifugation). Six market weight crossbred lambs were slaughtered and a portion of the longissimus lumborum was removed at death (within 15 min of exsanguination) and after 3, 6, 9, 12, 24, 72, and 360 h postmortem. Muscle samples were snap-frozen in liquid nitrogen and stored at −70°C. Soluble muscle proteins were extracted from muscle samples and analyzed by in-gel casein assay to measure calpain proteolytic activity. There was a gradual decline in μ-calpain activity ($P < 0.05$) such that after 24 and 72 h postmortem, μ-calpain had lost 42 and 95% of its activity, respectively. After 360 h postmortem, no μ-calpain activity could be detected (under the conditions used in this study). Autolysis of μ-calpain could be detected as early as 3 h postmortem. It was demonstrated that the detectable level of μ-calpain activity is a function of the amount of muscle protein electrophoresed. Hence, the activity data reported are in relative terms, rather than absolute values. Furthermore, it was demonstrated that the activity data also are a function of the assay methods used. Different methods have different lower detection limits. Of the three assays examined, ^{14}C-labeled casein was the most sensitive, then the in-gel casein assay, and the least-sensitive method was the standard casein assay. Unlike μ-calpain, postmortem storage had no effect on m-calpain ($P > 0.05$). When the calcium concentration of a muscle extract was increased to the level that induces m-calpain autolysis, m-calpain was autolyzed and its autolysis was readily detected by the in-gel casein assay. Collectively, these results demonstrate that calcium concentration in postmortem muscle is only high enough to activate μ-calpain. These results support the widely believed conclusion that μ-calpain-mediated proteolysis of key myofibrillar and cytoskeletal proteins is responsible for postmortem tenderization. Hence, understanding the regulation of μ-calpain in postmortem muscle should be the focus of future studies.

Key Words: Calpains, Meat, Postmortem Changes, Tenderizing

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Introduction

Current evidence suggests that postmortem meat tenderization is primarily the result of calpain-mediated degradation of key myofibrillar and cytoskeletal proteins (for reviews, see Goll et al., 1991; Koohmaraie 1992b, 1996). The result of the degradation of these proteins is that myofiber linkages are broken, causing weakening of the muscle structure, and, therefore, meat becomes tender.

Although there seems to be general agreement that calpain is the proteolytic system that causes postmortem proteolysis and tenderization, it is not clear whether both μ- and m-calpain are involved in the process or whether μ-calpain alone is responsible (Koohmaraie, 1996; Boehm et al., 1998). Koohmaraie et al. (1987) reported that μ-calpain and calpastatin gradually lost their activity with postmortem storage, but m-calpain was very stable and did not lose activity. Most of the studies that have used appropriate methodology (Veiseth and Koohmaraie, 2000) have found the same effect of postmortem storage on the activity of the components of the calpain system (e.g., Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1991). Because m-calpain is very stable and μ-calpain is not and because exposure to calcium leads to inactivation of both μ-calpain and m-calpain, Koohmaraie et al. (1987)

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suggested that μ-calpain rather than m-calpain was responsible for postmortem proteolysis that results in meat tenderization. Koohmaraie et al. (1987) suggested that autolysis is the mechanism of inactivation of μ-calpain in postmortem muscle. Unlike autolysis of μ-calpain, SDS-PAGE or Western blotting cannot detect autolysis-induced changes in the mass of m-calpain. The objective of this experiment was to use casein zymography (Raser et al., 1995) to better assess the status of the calpains in postmortem muscle.

Materials and Methods

Animals

The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study. Six crossbred lambs (Dorset × Romanov) were slaughtered and a portion of the longissimus was removed at 0, 3, 6, 9, 12, 24, 72, and 360 h postmortem. The portions were trimmed of visible fat and connective tissue, cut into small pieces, snap-frozen in liquid nitrogen, and stored at –70°C for 4 to 6 mo.

Sample Preparation

After 4 to 6 mo of storage, 300 mg of muscle were homogenized in 3 vol of extraction buffer (100 m*M* Tris base, 10 m*M* EDTA, 0.05% 2-mercaptoethanol [**MCE**], pH 8.3) using a Polytron on speed setting 6 (Brinkman Instruments, Westbury, NY), three times for 15 s, with a 15-s cooling period between bursts. The homogenate was centrifuged at 8,800 × g_{max} for 30 min, and the volume of the supernate was measured.

Casein Zymography

Casein zymography was based on the protocol described by Raser et al. (1995). Casein (0.21%, wt/vol) was incorporated in 12.5% separating gels (75:1 ratio of acrylamide to bisacrylamide, 375 m*M* Tris·HCl, pH 8.8), and 4% gels (75:1, 125 m*M* Tris·HCl, pH 6.8) without casein were used as stacking gels. TEMED (0.05%, vol/vol) and ammonium persulfate (0.05%, vol/vol) were used to catalyze the polymerization. Sample buffer (150 m*M* Tris·HCl, pH 6.8, 20% glycerol, 0.75% MCE, 0.02% [wt/vol] bromophenol blue) was added to supernate equivalent of 4 mg of muscle, so that final volume was 20 μL and final concentration of Tris was higher than 20 m*M*. The casein minigels (0.75 mm) were prerun at 100 V for 15 min, 4°C, with a running buffer containing 25 m*M* Tris·HCl, 0.05% MCE, 192 m*M* glycine, and 1 m*M* EDTA (pH 8.3) before samples were loaded into the wells. The gels were run at 100 V for 8 h, 4°C, removed, and incubated at room temperature in 50 m*M* Tris·HCl, 0.05% MCE and 4 m*M* $CaCl_2$ (pH 7.5) with slow shaking for 1 h (three changes of buffer). This was followed by a 16-h incubation in the same buffer at room temperature, before staining for 1 h with Coomassie blue (R-250) and destaining with 20% methanol and 7% acetic acid for 2 h; SE 500 casein gels (14- × 8.5-cm slab gel, 0.75 mm; Hoeffer Scientific Instruments, San Francisco, CA) followed the same protocol, except that they were run for 16 h.

Images were taken with a ChemiImager 4000 digital imaging analysis system (Alpha Innotech Corp., San Leandro, CA). Each gel included an independent at-death ovine muscle sample run in both of the outer lanes and in the middle lane as a reference standard. This standard was extracted each time samples were extracted. The quantification of μ- and m-calpain was done by expressing the density of the bands from the samples relative to the mean density of the reference standards within each gel.

Titration of a 72-h Postmortem Sample

A 72-h postmortem sample from one lamb was prepared as described above. The supernate was mixed with sample buffer and volumes equivalent to 16, 8, 4, 2, 1 and 0.5 mg of muscle were loaded on an SE 500 casein gel for detection of μ-calpain and m-calpain.

Autolysis of m-Calpain

Snap-frozen ovine longissimus muscle, at 360 h postmortem, was homogenized and centrifuged as described above. To induce m-calpain autolysis, an aliquot of the supernate was adjusted to 0.5% MCE and 5 m*M* $CaCl_2$ and incubated in a water bath at 25°C for 0, ½, 1, 2, 5, 10, 15, and 30 min. Autolysis was stopped by adding EDTA to a final concentration of 28.5 m*M*. Aliquots equivalent to 4 mg of muscle were mixed with sample buffer and run on casein minigels as described above.

Assay Sensitivity

μ-Calpain (0.5 unit of activity; after chromatographic separation on DEAE-Sephacel) was serially diluted to 1:256 (0, 1:1, 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256) with elution buffer and assayed using the standard protocol of Koohmaraie (1990); and the [^{14}C]casein assay of Koohmaraie (1992a) with slight changes. The reaction consisted of 100 μL of sample, 10 μL of 100 m*M* $CaCl_2$ and 25 μL of [^{14}C]casein. After 1 h incubation at 25°C, the reaction was stopped by first adding 100 μL of 10 mg/mL BSA (carrier) followed by protein precipitation induced by adding 300 μL of 10% cold trichloroacetic acid (**TCA**). After centrifugation at 8,800 × *g* for 15 min, half of the final volume (267 μL) was taken out of the TCA-soluble fraction and radioactivity was determined with a Packard model 460 liquid scintillation counter (Packard Instrument, Meriden, CT). The dilutions were also assayed with casein zymography. The diluted samples (85 μL) were mixed with 15 μL of sample buffer, loaded on SE 500 casein gels, and run as described above.

Table 1. Caseinolytic activity* of μ-calpain and m-calpain at different postmortem times (n = 6)

Time postmortem, h	μ-calpain activity	m-calpain activity
0	130.9[a]	104.1
3	124.3[ab]	104.9
6	109.1[bc]	106.3
9	98.1[c]	103.0
12	79.2[d]	103.6
24	75.6[d]	104.6
72	7.1[e]	103.7
360	N.D.[e]	103.8

*Each gel included an independent at-death ovine muscle sample run in both of the outer lanes and in the middle lane as a reference standard. This standard was extracted each time samples were extracted. The quantification of μ- and m-calpain was done by expressing the density of the bands from the samples relative to the mean density of the reference standard within each gel.

[a,b,c,d,e]Means in a column without a common superscript differ ($P < 0.05$). SEM for μ-calpain activity was 5.8. SEM for m-calpain activity was 1.8.

N.D. = Not detectable.

Statistical Analysis

Data were analyzed by analysis of variance for a repeated measures design (SAS Inst. Inc., Cary, NC). The main effect was postmortem time (0, 3, 6, 9, 12, 24, 72, and 360 h). Mean separation for significant ($P < 0.05$) time effects was accomplished by the PDIFF option (a pairwise *t*-test) of the least squares means procedures (SAS Inst. Inc.).

Results

Results presented in Table 1 and Figure 1 indicate that, whereas μ-calpain gradually lost its activity, m-calpain was very stable during a 15-d postmortem storage period. Results presented in Table 1 and Figure 1 indicate that, on average, 0- and 3-h μ-calpain activities were not different, but in some animals μ-calpain may have been activated as early as 3 h postmortem (Figure 1). To verify this observation, casein zymography was conducted at various times postmortem on a large slab gel (Figure 2). Although minigels are adequate for quantification purposes, large slab gels proved more informative for detecting changes in μ-calpain. Results presented in Figure 1 show that at 3 h postmortem we could detect the appearance of a second polypeptide, presumably the product of μ-calpain autolysis. The data presented in Table 1 indicate that the mean μ-calpain activity at 72 h postmortem was 5.4% of that of at-death μ-calpain. It is important to emphasize that 5.4% is the mean value of the lambs in this study and that the range in 72-h μ-calpain activity was from not detectable to 21.9%. Furthermore, it is important to use these values in the context of the sensitivity of the assay used (Koohmaraie, 1996). To demonstrate this point, we conducted the casein gel assay on 72-h longissimus of one lamb at various protein loads (Figure 3). At the lowest protein concentration, the amount of μ-calpain is just above the detection limit (see below) of the assay. However, at higher loads, μ-calpain activity could readily be detected. Therefore, if we had loaded more protein for 72-h and 360-h samples, although the percentage activity might not have changed, the absolute values would have increased.

We could not detect any decline in the level of m-calpain during the 15-d storage period (Table 1). To our knowledge, casein zymography of the autolyzed m-calpain has not been reported. It is well known that

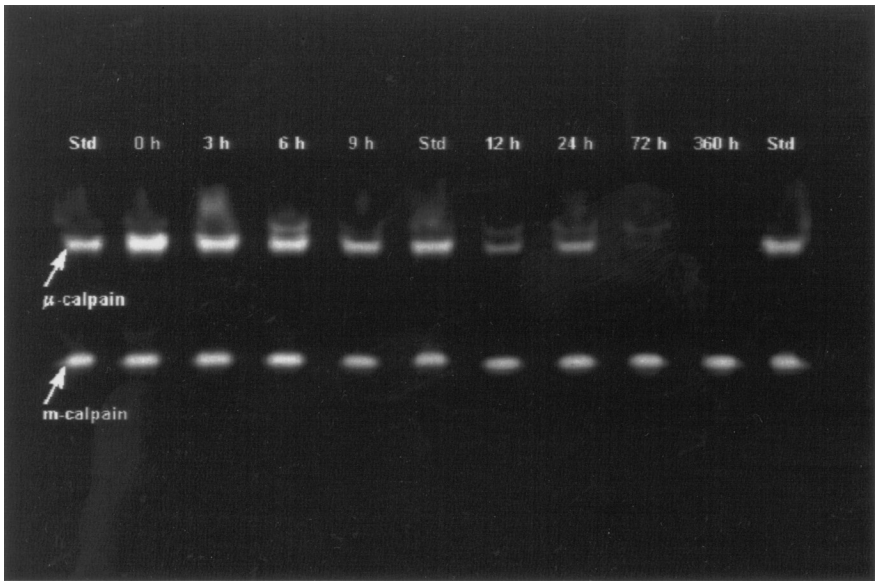

Figure 1. Casein gel analysis of lamb longissimus at various times postmortem at 4°C. Samples from all lambs were analyzed using minislab gels. The standard is an independent at-death ovine muscle; this standard was extracted each time samples were extracted. μ-Calpain and m-calpain were quantified by expressing the density of the bands from the samples relative to the mean density of the standard.

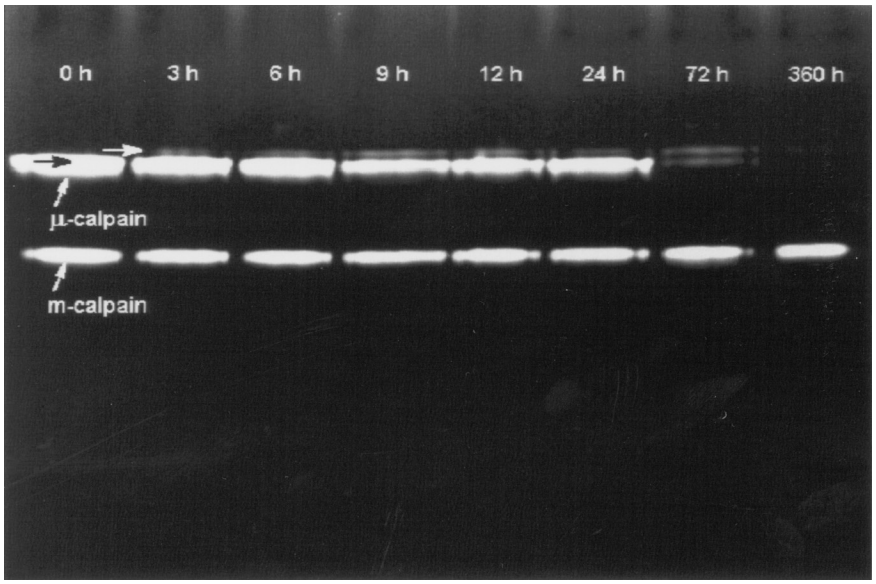

Figure 2. Casein gel analysis of the same sample as in Figure 1, but on a large slab gel, 8.5 cm × 14 cm. The black horizontal arrow indicates native μ-calpain and white horizontal arrow indicates autolyzed μ-calpain.

changes in the molecular mass of the large subunit of the m-calpain cannot be detected by SDS-PAGE. Therefore, we selected the longissimus of a lamb at 15 d postmortem that had no detectable μ-calpain activity (using casein zymography), and prepared a muscle extract as described for casein zymography. To induce m-calpain autolysis, the calcium concentration of the extract was increased to 5 m*M* and aliquots were removed at various times of incubation and analyzed by casein zymography. Casein gel analysis clearly demonstrated m-calpain autolysis after 30 s of incubation (Figure 4). These results demonstrate that if autolysis of m-calpain had occurred in postmortem muscle, we would have been able to detect it. Therefore, it is very unlikely that m-calpain is exposed to sufficient calcium in postmortem muscle to express its proteolytic activity.

We also determined the detection limits for calpain activity of casein zymography, the standard casein assay (Koohmaraie, 1990), and ^{14}C-labeled casein assay (Koohmaraie, 1992a). The lower detection limit for the standard casein assay is about 0.06 optical density (OD) (Figure 5). The lower detection limit for the casein gel assay was integrated density value of 1,800, which corresponds to 0.0075 OD of the standard casein assay. Therefore, casein gel assay is about eight times more sensitive than the regular casein assay. The lower de-

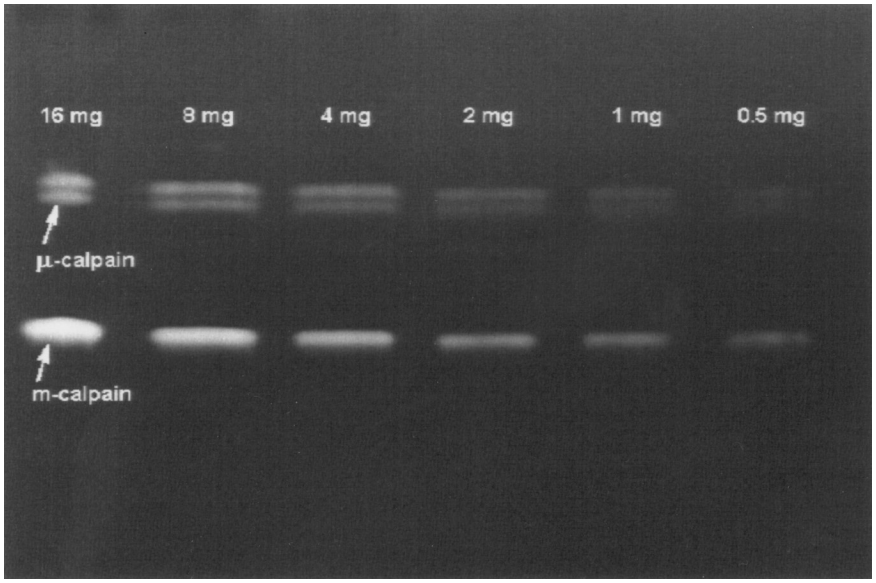

Figure 3. Casein gel analysis of 72-h postmortem lamb longissimus at various protein loads.

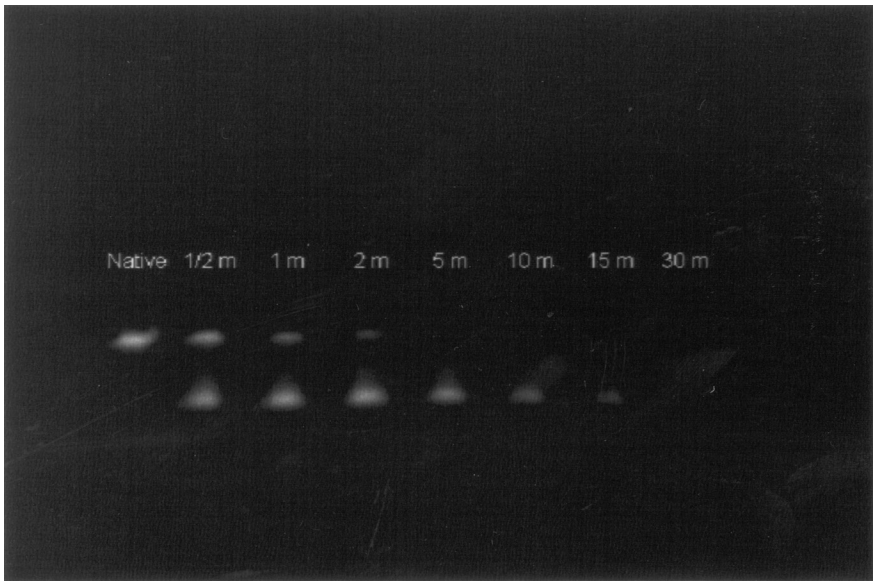

Figure 4. Casein gel analysis of m-calpain autolysis. Soluble fraction of a d-15 postmortem lamb longissimus was brought to 5 m*M* $CaCl_2$ and at various times sampled for analysis by casein gel.

tection limit for the ^{14}C-labeled casein assay (when the specific activity was 1,630 cpm/μg casein) was 315 cpm (the lowest dilution used), which corresponds to 0.94×10^{-3} OD of the regular casein assay. Therefore, ^{14}C-labeled casein assay is about 8 times more sensitive than the casein gel assay and about 64 more times more sensitive than the regular casein assay. Obviously, the sensitivity of the ^{14}C-labeled casein assay depends on

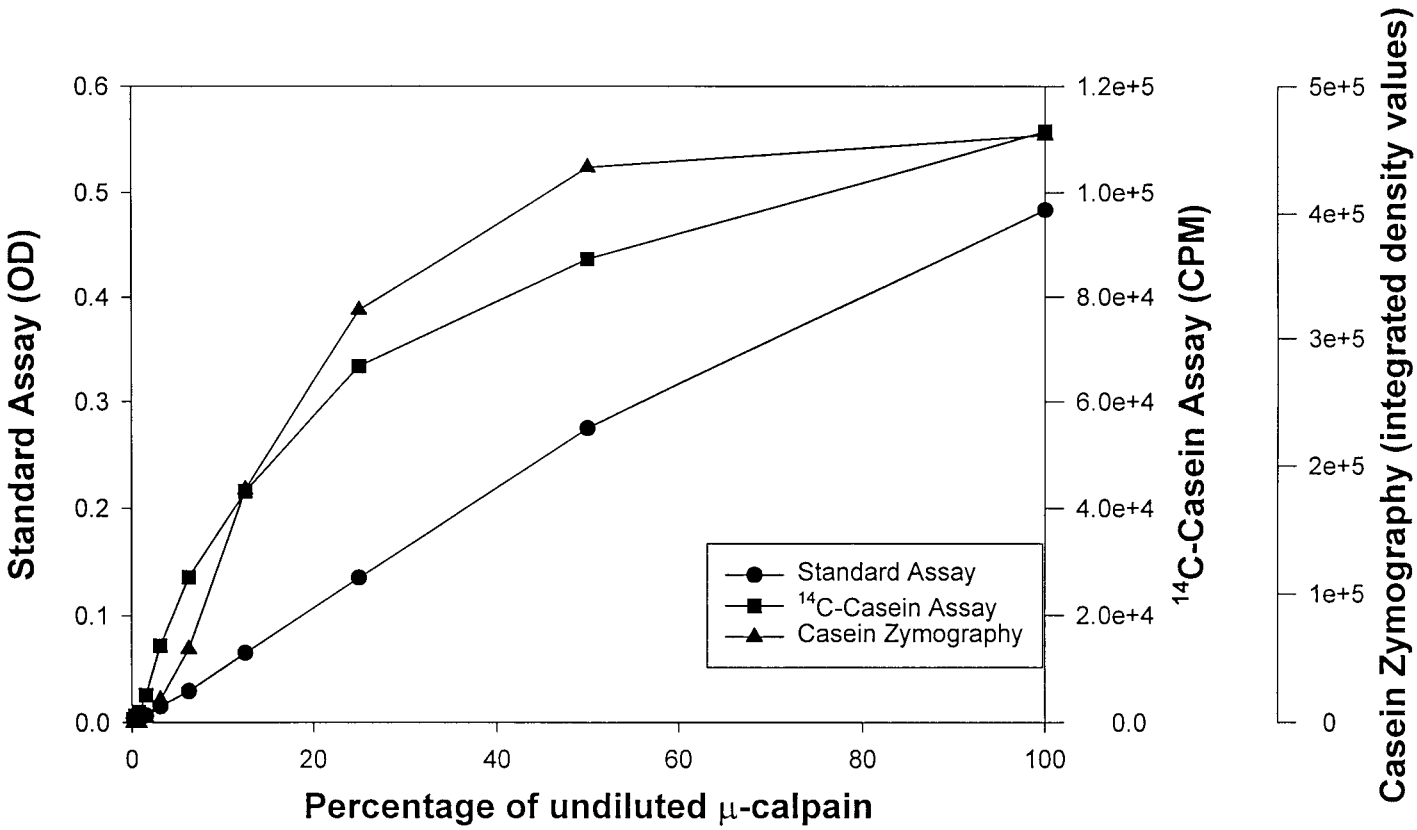

Figure 5. Assay of μ-calpain with three different methods. DEAE-Sephacel-purified porcine triceps brachii μ-calpain was diluted from 0 to 1:256 and assayed with standard casein assay, [^{14}C]casein assay, and casein gel assay.

the level of ^{14}C incorporation (which can be controlled) and hence it can be far more sensitive than the level stated here. Likewise, the casein gel assay could be modified to be far more sensitive by using flourescent zymograms. Casein can be labeled with fluorescein isothiocyanate (Simon et al., 2000) or other fluorogenic compounds, such as BODIPY. BODIPY-Casein assay is 100 times more sensitive than the standard assay (Geesink and Koohmaraie, unpublished data). However, our purpose was to demonstrate the limitation of each assay and to indicate that the ability to detect the activity of μ-calpain at any time postmortem, to a large extent, is a function of the assay used. For example, while using the standard casein assay, many studies have reported that μ-calpain has very little to no activity beyond the first 2 to 3 d postmortem. Using ^{14}C-labeled casein assay, Geesink and Koohmaraie (1999a,b) were able to demonstrate that ovine skeletal muscle contained active μ-calpain even after 56 d postmortem (2.5% of at-death μ-calpain activity).

Discussion

A unique property of the calpains is that, when exposed to sufficient calcium, they undergo autolysis. Autolysis-induced changes in the 80-kDa subunit of the μ-calpain are readily detectable by SDS-PAGE and Western blotting. At low ionic strength and sufficient calcium concentration, autolysis initially reduces the mass of the 80-kDa subunit of μ-calpain to 76 kDa, through a 78-kDa intermediate, and the mass of the 28-kDa subunit to 18 kDa. This partial autolysis reduces the calcium requirement for proteolytic activity but does not affect its specific activity. Further autolysis leads to more extensive degradation of the large subunit and loss of proteolytic activity (Inomata et al., 1986; Edmunds et al., 1991; Koohmaraie, 1992a). Because the autolysis of μ-calpain is an intermolecular process, autolysis does not result in complete loss of proteolytic activity of μ-calpain (Inomata et al., 1988; Cottin et al., 1991; Edmunds et al., 1991). At high ionic strength, autolyzed μ-calpain is unstable, and as a result the 80-kDa subunit is reduced to 78 and 76 kDa only (Geesink and Koohmaraie, 1999b, 2000). Autolysis of m-calpain at low ionic strength has a similar effect as for μ-calpain. Because autolysis of m-calpain is both an intermolecular and an intramolecular process, autolysis results in complete loss of proteolytic activity of m-calpain (Inomata et al., 1988; Cottin et al., 1991; Edmunds et al., 1991).

The effect of postmortem storage on the activity of the components of the calpain proteolytic system is well documented (for review, see Koohmaraie 1994, 1996; Geesink and Koohmaraie, 1999a,b). Because 1) m-calpain is very stable and μ-calpain is not, 2) exposure to calcium leads to inactivation of both μ-calpain and m-calpain, and 3) intracellular free-calcium concentration in postmortem muscle is too low for expression of m-calpain proteolytic activity, Koohmaraie et al. (1987) suggested that μ-calpain rather than m-calpain is responsible for postmortem proteolysis that results in meat tenderization. Koohmaraie et al. (1987) suggested that extensive autolysis to small and proteolytically inactive polypeptides is the mechanism of inactivation of μ-calpain in postmortem muscle. However, Geesink and Koohmaraie (1999b, 2000) demonstrated that autolyzed μ-calpain is very unstable at postmortem ionic strength and that instability of autolyzed μ-calpain is a major cause for the decline in μ-calpain activity in postmortem muscle. They modified the conclusion of Koohmaraie et al. (1987) by concluding that autolysis and instability of the autolyzed enzyme lead to inactivation of μ-calpain in postmortem muscle.

Because the 28-kDa subunit is identical in both calpains and because μ-calpain is autolyzed postmortem, autolysis of the 28-kDa subunit cannot be used to study postmortem effects on m-calpain. N-Terminal sequencing of m-calpain from at-death 7-d postmortem muscle failed, which indicates the 80-kDa subunit of the m-calpain is not autolyzed (Boehm et al., 1998). The N-terminal sequencing information could be sufficient evidence for stability of m-calpain (inactive) in postmortem muscle. Because m-calpain had to be purified for N-terminus sequencing (Boehm et al., 1998) and because autolyzed m-calpain is unstable during ion-exchange chromatography (Suzuki et al., 1981; Koohmaraie et al., 1989; Edmunds et al., 1991), the purification protocol used by Boehm et al. (1998) will not isolate autolyzed m-calpain. Hence, N-terminus sequencing alone cannot be used to determine the status of the m-calpain in postmortem muscle.

We used casein zymography to assess the status of m-calpain in postmortem muscle. Details about casein zymography can be found elsewhere (Raser et al., 1995; Simon et al., 2000; Zhao et al., 2000). Briefly, muscle extracts are run on nondenaturing gels in the presence of EDTA (to prevent calpain autolysis). Casein (calpain substrate) is copolymerized with polyacrylamide. After completion of electrophoresis and washing, the gel is incubated with calcium and a reducing agent to activate calpains. Gels are then stained with Coomassie brilliant blue. Clear areas on the gel are indicative of calpain activity. Combined, our results demonstrate that, unlike μ-calpain, m-calpain is very stable in postmortem muscle. Results presented in Table 1 and Figure 1 indicate that beginning at 3 h postmortem, we can detect changes in μ-calpain activity. These results indicate that at about 3 h postmortem, calcium concentration is high enough to induce μ-calpain autolysis (Figure 2). Although the method is sensitive enough to detect autolysis of m-calpain (Figure 4), we could not detect any changes in m-calpain during the 15-d postmortem storage. Combining these results with results of other studies, we conclude that although calcium concentration in postmortem muscle is high enough to activate μ-calpain, the calcium concentration is not high enough to activate m-calpain. Furthermore, these results support the widely believed conclusion that μ-calpain-me-

diated proteolysis of key myofibrillar and cytoskeletal proteins is responsible for postmortem tenderization.

Implications

These results indicate that of the two calpain isoforms only μ-calpain is active under postmortem conditions. Based on these and the results of numerous other studies, we conclude that the μ-calpain-mediated proteolysis of key myofibrillar and cytoskeletal proteins is responsible for postmortem tenderization. Hence, understanding the regulation of μ-calpain in postmortem muscle should be the focus of future studies. Such information can be used to optimize postmortem conditions to the extent possible to maximize the activity of μ-calpain and, hence, the rate and extent of postmortem tenderization.

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